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Arachidonic acid-induced degradation of articular cartilage

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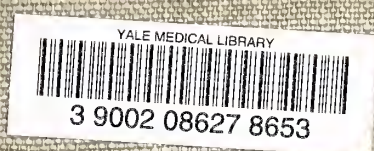
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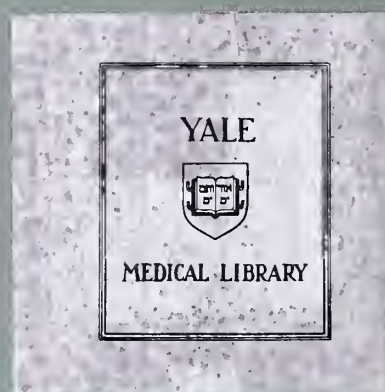
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Arachidonic Acid-induced Degradation of
Articular Cartilage.

by

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B. S., University of California

at Los Angeles,

1975.

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School of Medicine in Partial Fulfillment
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To Suzanne.

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Introduction

When one considers the stresses to which we subject the weight-bearing joints of our body over the course of a lifetime, one begins to appreciate the remarkable dependability and durability of that very thin layer of articular cartilage which covers the surfaces of those joints. Imposed on these surfaces are variety of constant and cyclic loads, stemming from forces which are both compressive and shearing. Yet, this thin layer stands up to year after year of load-bearing, with its own system of lubrication and replenishment of worn material, an achievement which cannot be boasted by any man-made mechanical device.

Unfortunately, nature itself is not free from imperfections and this wondrous mechanical system often fails. A small mechanical breakdown, a slight biochemical change often leads to a chain of events which results in pain and incapacitation. It is estimated that some 40 million Americans manifest radiological evidence of such joint degeneration, including some 85% of all those over 70 years of age and at a cost of \$13 billion in 1976.

This paper will briefly discuss some of the pertinent properties of articular cartilage and degenerative joint disease (osteoarthritis). It will then

turn to some of the current ideas concerning the biochemical processes which may be responsible for joint failure and some of the experiments performed here to help elucidate one of these ideas: that the joint disease is mediated through the production of prostaglandins within the joint.

Articular Cartilage

Development

Joint development begins during the fifth to sixth week of embryonic life with the appearance of limb buds (Langman 1975). Soon thereafter, clefts appear in the appendages, forming joints. At birth, endochondral bone formation occurs at the epiphyses. The articular cartilage may be considered as a partial spherical shell which covers the cartilage surrounding the ossification center. This superficial shell has a separate population of chondrocytes which lay down the articular cartilage. It ceases to proliferate at about the first year of life (Mankin 1963).

Upon skeletal maturity and the appearance of the tidemark (seen on hematoxylin-eosin staining), no further mitosis occurs (mankin 1963, Crelin 1957). However, under appropriate stimulation, the chondrocytes can revert to a chondroblastic form with DNA production, mitosis and increased mucopolysaccharide production. These stimuli may be growth hormone (Chrisman 1975), sustained compression (Crelin & Southwick 1960), laceration (Meachim 1963) and osteoarthritic destruction (McDevitt 1973). Unfortunately, in the last case, the new proteoglycans are either not produced in sufficient quantities or are of poorer quality (McDevitt 1973) and destruction is not completely reversed.

Biochemistry

Besides the chondrocytes, articular cartilage consists mostly of matrix made up of 3 principal components: water, collagen and proteoglycans.

Water: Seventy five to 85% of the weight of articular cartilage is comprised of water (Mankin & Thrasher 1975). Of this, about 94% is mechanically trapped in a complex gel of collagen and proteoglycan and is freely exchangeable with both the synovial fluid and systemic circulation (Jaffe, et al 1974).

Collagen: Collagen fibrils are aggregates of tropocollagen molecules. Tropocollagen is usually a triple helix with 2 identical α_1 chains and a somewhat different α_2 chain (Muir 1873). For example, skin collagen is a mixture of molecules of compositions $[\alpha_1(1)]_2\alpha_2$ and $[\alpha_1(111)]_3$ (McDevitt 1973). Articular cartilage tropocollagen consists of 3 identical strands of $\alpha_1(11)$ chains, which has properties very similar to the $\alpha_1(1)$ chain from skin (Strawich & Nimmi 1971). It should be noted that articular cartilage collagen is almost completely resistant to extraction by reagents commonly used for extraction of skin cartilage, such as 5 M guanidine-HCl (Miller, et al 1969), 2 M $MgCl_2$ or 6 M urea (Strawich & Nimmi 1971). This has been attributed to both intra- and inter-molecular cross-linking and ionic interactions between the positively charged proteoglycans (McDevitt 1973).

The biocynthesis of collagen is now well-understood though the work has been done mostly with chick embryo cartilage (Muir 1973). The polypeptide chains are formed singly which then spontaneously aggregate to form a triple helix, at which stage, the molecule is referred to as procollagen. Hydroxylation of some of the proline and lysine residues then occurs (Rosenbloom & Prockop 1969). A different enzyme is involved in the hydroxylation of each (Weinstein, et al 1969). Some of the hydroxylated lysine residues are then glycoslated to either glycosyl-galactosyl-hydroxylysine or galactosyl-hydroxylysine (Blumenkrantz, et al 1969), before the tropocollagen molecule is secreted from the cell. In the cartilage matrix, the molecules aggregate to form fibrils, after which a slow maturational process of intra- and intermolecular cross-linking occurs (Muir 1973).

There is a slow, but detectable level of collagen turnover in adult rabbit articular cartilage which increases with experimentally-induced osteoarthritis (Repo & Mitchell 1971).

Proteoglycans: Proteoglycans are large molecules which structurally consist of a protein core from which are hung glycosaminoglycan side chains, made of 3 major disaccharides: chondroitin-4-sulphate, chondroitin-6-sulphate and keratan sulfate, the structures of which are shown in Figure 1. Chondroitin-4-sulfate has a

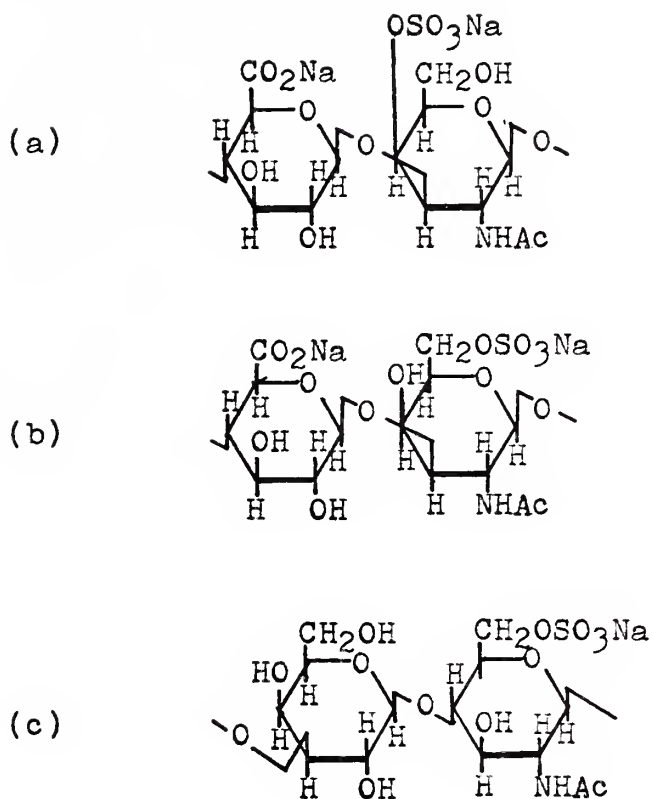


Figure 1: Disaccharide repeating units of (a) chondroitin 4-sulphate, (b) chondroitin 6-sulphate and (c) keratan sulphate.
(From Freeman M.A.R. 1974)

disaccharide unit consisting of glucuronic acid linked to N-acetyl galactosamine, with the sulphate group at the galactose C⁴ position. In the case of chondroitin-6-sulphate, the sulphate is in the C⁶ position. In keratan sulphate, there is a galactose molecule linked to an N-acetyl glucosamine-6-sulphate molecule. The chondroitin sulphate chains tend to be linear, with 30 to 60 disaccharide units, while that of keratan sulphate may be more variable, with 15 to 30 disaccharide units per chain with some branch points (Hopwood & Robinson 1974). Moreover, keratan sulphate also contains small amounts of other sugars such as fucose, sialate, galactosamine and mannose (Mathews & Cifonelli 1965). The chondroitin sulphate chains do not appear to be distributed uniformly along the protein core but are present in pairs (Mathews 1971), with about 10 amino acid residues between individual chains and about 35 residues between pairs. It is believed that the protein core helps to protect the polysaccharides from enzymatic attack by holding them closely together (Muir 1973). This is consistent with the observation that proteoglycans are more susceptible to attack by proteolytic enzymes than hyaluronidases (Cessi & Bernardi 1965). The relative proportions of the glycosaminoglycans change over life. The concentration of keratan sulphate increases, that of chondroitin-4-sulphate falls and that of chondroitin-6-sulphate remains constant (Barnett,

et al 1963)).

The proteoglycan monomer appears as a comb with the protein core acting as the "backbone" and the polysaccharide "teeth" hanging from it. Rosenberg has postulated that the C terminals of the polypeptides associate to form a dimer. Dimers then aggregate around a link protein to form starlike first-order aggregates. The free N terminals of some of the "arms" of the stars can further associate with link proteins of other first-order aggregates to form second order aggregates (clusters of stars) (Rosenberg, et al 1970), although it is unclear that this actually occurs in vivo. In addition to the link protein, hyaluronic acid is necessary for aggregation, and the present evidence suggests that it interacts directly with one part of the link protein (Hardingham & Muir 1972, 1973, Kuettner, et al 1975). Several "stars" are believed to be strung out along a long strand of hyaluronic acid. This extended structure stems from the mutually repulsive forces of the negatively-charged polysaccharide side-chains, resulting in their occupation of the greatest amount of space. This electrostatic interaction by itself contributes to cartilage elasticity. This structure also allows it to retain large quantities of water, and gives it the ability to absorb stresses placed on the cartilage by increasing the viscosity of the cartilage matrix (McDevitt 1973). This shock-absorbing quality is unique to cartilage.

The proteoglycans are synthesized by the chondrocytes. The polypeptide chain is made on the ribosomes. The disaccharides are formed separately and are then polymerized at the appropriate linkage sites on the polypeptide (Horwitz & Dorfman 1968). Sulphation occurs both in the rough endoplasmic reticulum and Golgi apparatus (Martinez, et al 1977). The molecules are then packaged, also in the Golgi apparatus, and released.

Some form of feedback regulation appears to be in operation. Chondrocytes have been shown both in vitro (Bosmann 1968) and in vivo (Edwards & Michael 1977) to respond to depletion of proteoglycan by increasing production.

Proteoglycans are degraded by both lysosomal and non-lysosomal enzymes present in the cartilage (Chrisman & Fessel 1962, Ali 1964). Using $^{35}\text{S-SO}_4$, Maroudas (1975) was able to measure the half life of cartilage. She found that the $T_{\frac{1}{2}}$ of the femoral head cartilage was 800 days while that of the femoral condyle cartilage was 300 days. She also found that differences occurred between different depths of the cartilage. Hall, et al (1977), on the other hand, found that although most of the proteoglycan in rabbits had a life-span of 600 days, 9.5% of it had a half life of only 6.8 days, implying an active and inactive pool of proteoglycans. Turnover of the glycosaminoglycans occurs at different rates, with keratan sulphate being turned over

faster than the chondroitin sulphates.

There is variation in the concentration of glycosaminoglycans both depending on the depth (Lipshitz, et al 1976) and the topographical location (Bjelle 1974). An increase in concentration with depth occurs, up to about 35% of the cartilage depth, followed by a gradual decline (Lipshitz, et al 1976). Bjelle (1974) showed significant differences in the hexosamine concentrations in different locations of the femoral side of the knee joint. There is also considerable variation from one person to the next (Maroudas 1975).

The negative charges of the sulphate and carboxylate groups combine with many dyes which allow the hexosamines to be identified histochemically. Safranin-O, for example, can be used to detect the glycosaminoglycans semi-quantitatively (Rosenberg 1971). Under proper conditions, the dye binds stoichiometrically to the negatively charged groups. There is no interference from the collagen.

Architecture

Classically, using techniques of light microscopy, the architecture of articular cartilage consists of the following zones proceeding from superficial to deep: the gliding zone, the transitional zone, the radial zone, the tidemark and the calcified zone, which lies over the subchondral bone. Recent ultrastructural work

has increased our knowledge of the histology but the names of the basic zones may be largely retained.

a. The gliding zone: This is subdivided into two layers: the superficial and tangential layers. The superficial layer is about 200 microns thick and consists only of fine collagen fibrils arranged randomly (Redler 1974). There is no proteoglycan in this layer. In the tangential layer, collagen fibers are arranged in a plane parallel to that of joint motion (Redler 1974). Cells in this layer resemble fibroblasts with their long axes in a tangential plane. It has been found that the superficial layers have a higher tensile strength to forces parallel rather than perpendicular, to the direction of joint motion (Woo, et al 1976), a finding consistent with the ultrastructural observations. This combination of fine fibrils with underlying fibers is seen by the scanning electron microscope as a series of gently undulating ridges and furrows (Redler 1974). It is proposed that this topographical arrangement serves as a means of facilitating joint lubrication by allowing collection of synovial fluid (Chrisman 1978). The tangential orientation of the collagen fibers is also ideal for resisting destruction due to the shearing forces of joint articulation.

b. The transitional zone: The ratio of cells to matrix decreases as the depth increases. The chondrocytes tend to be in spherical lacunae, separated by matrix

containing water, collagen and proteoglycan. Some of the collagen bundles are no longer tangential but are oblique.

c. The radial zone: Deep to the transitional zone, a zone of more radially oriented collagen fibers are encountered. The chondrocytes are seen under the light microscope to be lined up in columns. Some of the radial bundles form arcade-like patterns (Hunter & Finley 1973) although the arches are not formed by continuous fibers. Deep in the zone, the bundles tend to anastomose into large bundles and merge into the calcified zone (Redler, et al 1975). The radial arrangement provides the cartilage with strength against compressional stresses, as in joint load-bearing. This zone is also particularly rich in proteoglycans and water, which gives it the elasticity and added stiffness (Meachim & Stockwell 1973).

d. The tidemark: This is an undulating, hematoxylin-staining line of 2 to 5 microns in thickness, which demarcates the calcified and uncalcified portions of articular cartilage. There are 3 bands discernible: (1) a band of randomly-oriented fibrils continuous with those of each side of the tidemark, (2) a band of fibrils parallel to the surface of the calcified cartilage and (3) a band of fibrils oriented perpendicular to that surface. It has been suggested that this arrangement serves as a tethering mechanism for the flexible radial

fibers of the radial zone and prevents them from being sheared off by tangential stresses at their points of anchorage to calcified cartilage (Redler, et al 1975).

e. The calcified zone: Calcification of this zone increases with skeletal maturity. In immature cartilage, there are short columns of chondrocytes separated by matrix. Vascular structures are present, with calcification most advanced in the perivascular areas. Calcification continues to progress upon skeletal maturity, with loss of the columnar organization of the chondrocytes. The function of the calcified zone is to provide the non-calcified cartilage with a firm attachment to the sub-chondral bone (Meachim & Stockwell 1973), with which it has a markedly undulating interface.

f. The subchondral bone: This is not part of the articular cartilage per se. There is an endplate consisting of dense cortical bone which supports the cartilage. In turn it is supported by trabecular bone. It is believed that the trabeculae have shock-absorptive properties.

Osteoarthritis

Osteoarthritis (degenerative joint disease, hypertrophic arthritis) is a condition which afflicts a large segment of the older population. In young people, osteoarthritis may occur as a result of trauma, septic arthritis or congenital abnormalities of either a biochemical or morphological nature (secondary form). The exact etiology and pathogenesis of osteoarthritis, especially that which afflicts the older age group (primary form), have not been completely worked out. However, some of the current ideas will be discussed later. There is at present no cure for it. Treatment is directed towards minimizing symptoms and incapacitation.

Clinical Manifestations

The most important complaint of patients with this condition is joint pain, especially with motion and weight bearing. There is also stiffness, but unlike rheumatoid arthritis, the pattern of prolonged morning stiffness, gradually subsiding, is usually absent. On physical examination, there is often decreased range of motion, tenderness to palpation with perhaps a small effusion, some bone enlargement and crepitus. Markedly warm and erythematous joints are unusual. (Mannik & Gilliland 1977).

When the distal interphalangeal joints of the

fingers are involved, bony nodules at the base of the distal phalanges (Heberden's nodes) are found, the corresponding lesions of the proximal interphalangeal joints are called Bouchard's nodes. Wrists, elbows and shoulders are uncommonly affected.

Osteoarthritis of the spine usually causes back pain and stiffness. Radiologically, osteophytes are readily seen although most cases are asymptomatic. They may encroach upon the spinal foramina and cause nerve root compression giving rise to radicular pain, weakness of affected muscle groups and even muscle atrophy. In the cervical area, they may occlude the vertebral artery on turning the head, giving rise to transient visual disturbances or syncope.

Hip involvement is one of the most disabling. With severe involvement, patients may be completely unable to ambulate on their own. Patients complain of pain in the hips, groin or knees. Radiologically, there is often loss of joint space (indicating articular cartilage destruction) and bony thickening of the femoral heads. Secondary osteoarthritis of the hip usually occurs at an earlier age. The causes include trauma, fractures, septic arthritis, rheumatoid arthritis, any congenital abnormalities of the hip (dislocation, dysplasia), slipped capital femoral epiphysis and avascular necrosis (often associated with sickle cell disease, steroid therapy, etc.).

Trauma is usually the cause of osteoarthritis of the knee. Injuries to the menisci or supporting ligaments give rise to joint instability and abnormal wear and tear of the articular cartilage. Pain on weight-bearing and ambulation is the most common complaint.

Similarly, involvement of the ankles in osteoarthritis is often associated with trauma. Primary osteoarthritis of the ankles is uncommon.

It is of note that polyarticular osteoarthritis is a manifestation of acromegaly. There is a thickening of the synovial membranes and articular cartilage. As mentioned before (Chrisman 1975), growth hormone can cause mitosis and DNA production in "dormant" chondrocytes.

Diagnosis

The diagnosis is suggested by the age, symptoms, pattern of joint involvement and lack of accompanying abnormalities.

There is usually no elevation of the erythrocyte sedimentation rate. The joint fluid is often slightly turbid and may have a small amount of fibrin clot. The white cell count should be less than 2000 per mm³ with less than 25% polymorphonuclear cells. Higher white cell counts suggest the presence of the more inflammatory arthritides (rheumatoid, septic, etc.).

Radiological examination of the joints is very

useful for determining the extent of disease and for following its progress. Narrowing of the joint spaces, lipping, osteophytes are all consistent with but not diagnostic of osteoarthritis.

Treatment

Treatment of osteoarthritis is aimed at the relief of pain and the minimization of disability. General measures include a weight reduction program for over-weight individuals and a program of moderate exercise. A decrease in load-bearing on large joints helps decrease symptoms although no significant correlation between obesity and osteoarthritis has been found (Saville and Dickson 1968). The exercise program is aimed toward maintenance of joint mobility but heavy exercise should be avoided as further trauma can occur. The patient should be kept reassured that, in the majority of cases, osteoarthritis is neither fatal nor crippling (Howell, et al 1968).

The mainstay of drug therapy is aspirin at a dose of 2 to 4 gm. a day, if tolerated by the patient. For those who do not tolerate aspirin well, other potent anti-inflammatory and analgesic agents are available but they have their own problems of adverse effects. Indomethacin (75 to 100 mg. a day) is very effective in many patients as is phenylbutazone (200 to 400 mg. a day) but the latter drug should be used only for short

periods (1 week) due to its toxicity and should be reserved for treatment of acute exacerbations of symptoms.

Among those patients who do not respond well to medical management, a portion will benefit from surgical procedures which include osteotomies, arthroplasties and joint replacements. The hip joint has enjoyed the best results in total replacement, using a variety of prostheses, the most popular ones being those of Charnley (1961) and McKee and Farrar (McKee & Watson-Farrar 1966). Follow-up studies have been very impressive, with good to excellent success rates of these two methods being greater than 90% on follow-ups of up to 10 years (Charnley & Cupic 1973, McKee & Chen 1973). This has been considered a major advancement in the management of osteoarthritis of the hip and has spared many patients from pain and disability.

Pathology

The earliest pathological changes are chondromalacia (loss of proteoglycan from articular cartilage) and fissuring. The articular surface becomes slightly irregular (Robbins 1974). With progression of chondromalacia, there is an increase in the number of chondrocytes which are distributed in clusters ("cloning") (Mankin & Lippiello 1970). In addition, deep clefts or fissures form in the cartilage. The above processes are denoted by the term "fibrillation", which is considered

to be the hallmark of degenerative change in osteoarthritis (Collins 1949). Correlating this with the present ultrastructural data, one can conclude that as the superficial tangential layers lose proteoglycan, the layers are destroyed and are denuded. The underlying radial fibers are then exposed to the joint stresses and are in turn damaged along their lines of weakness, giving rise to histological fissuring. Eventually, continued wear and tear of the unprotected cartilage leads to massive denudation, which shows up radiologically as narrowing of the joint space.

The exposed underlying bone becomes thickened with new bone formation. In the marrow of the same region, small islands of cartilage of sometimes fibrous tissue (pseudocysts), are observed, which may have been either "driven in" or formed de novo (Robbins 1974). Some cartilage, especially that around the margins, ossify and project above the surface to form the characteristic spurs or osteophytes. The denuded surface of the bone itself becomes dense, smooth and glistening (eburnation) (Mannik & Gilliland 1976).

There is some thickening and inflammatory edema of the synovia, but this is low grade and does not have the vascularization or leukocytic infiltration found in the more highly inflammatory arthritides (Robbins 1974).

Pathogenesis

What causes the osteoarthritic changes? This question is more easily answered for the secondary forms than for the primary form.

It has long been held that osteoarthritis occurs as a consequence of trauma which results in some alteration of the joint anatomy. In recent years, with our greater awareness of the biochemical make-up of tissues, the definition of trauma was naturally broadened to cover any events which cause some microstructural or biochemical changes in the joint. Much work has been done in the induction of osteoarthritis by applying stresses to the joints under laboratory conditions.

The role played by mechanical trauma is obvious and well-documented. Among the many studies available is that Lane, et al (1978) who performed medial meniscectomies and transections of the medial collateral and both cruciate ligaments of rabbit knees. It was found that, although it took 2 months for gross histological changes to occur, it took only 1 day for detectable changes in the mechanical properties to take place. Presumably, osteoarthritis develops as a result of abnormal contact pressures. In osteoarthritis associated with burnt out rheumatoid arthritis, synovial and periarticular tissues proliferate and invade the articular cartilage (Mills 1970). The inflammatory

reaction resulting from a septic joint will induce the same changes.

The intra-articular presence of crystals in gout, pseudogout and ochronosis (Laskar & Sargison 1970) gives rise to abrasion of the articular surface and in addition can trigger off a foreign body reaction in the synovium (Sokoloff 1972).

Structural abnormalities secondary to conditions such as the dysplasias, Perthe's disease, fracture, dislocation and infarction fall into the broad category of mechanical trauma secondary to joint deformation.

In short, any event or condition which alters the make-up of the joint, be it anatomical, cellular or biochemical, will predispose that joint to develop osteoarthritic changes.

What about the more common primary or "idiopathic" form? Here, very little is known about the inciting or etiological event. It is widely, though not universally, believed that the first changes occur in the articular cartilage. There are those who propose that vascular changes in the underlying bone are the initial events (Arnoldi, et al 1972, Brookes & Helal 1968, Hulth & Hernborg 1968). Others propose that the initial event is the loss of resilience in the underlying bony trabeculae (Radin & Paul 1970). Without going into the controversies surrounding the different hypotheses, it is sufficient to say that most

workers agree that the initial events affect the articular cartilage (fibrillation). This begs the question regarding what leads to fibrillation. Putting together the fact that osteoarthritis tends to occur in old people and that any form of trauma can give rise to osteoarthritis, it is attractive to postulate that the inciting event in primary osteoarthritis is the damage accumulated from small doses of minor trauma during long-standing, normal use. Although attractive, this hypothesis is difficult, if not impossible, to prove, since there are no good laboratory or epidemiological models for "normal use".

One question has been whether osteoarthritic change represents an acceleration of normal ageing in articular cartilage. Comparison of autopsy material has shown that fibrillation can be found in the joints of unselected subjects (Meachim 1972), similar to that in osteoarthritic patients, but of a less advanced stage. On the other hand, Byers, et al (1970) looked at the location of these lesions and found significant differences between osteoarthritic and unselected cases.

As noted earlier, the composition of the proteoglycans changes with age. Could this be the initial event? Again, there is no data available.

In summary, the etiology of primary osteoarthritis is unknown. The notion of long-standing, normal use as being the cause is attractive but difficult

to prove scientifically.

Whatever the etiological factors are, the end result is the same. There is a loss of proteoglycans accompanied by a deterioration of the mechanical properties of the cartilage (Lane, et al 1978, McDevitt 1973). As the process of fibrillation advances, ulceration and erosion can be seen histologically.

How does this loss of proteoglycan take place? The presence of various species of degradative enzymes have been demonstrated in the synovial fluid (Lack & Ali 1967, Sapolsky, et al 1973, 1974, Ehrlich, et al 1975), the best known of which were the cathepsins, also found in rheumatoid synovia. However, it was later discovered that purified cathepsins have little proteolytic activity as the pH increases to 7 and that the significant tail-end of its activity at that pH was actually due to a contaminant in the previous cathepsin preparations (Woessner 1973, 1973a). By using known inhibitors of the cathepsins and various other enzymes of the synovia and leukocytes, Howell demonstrated that, at the pH of synovial fluid, proteolytic activity resided in a neutral protease derived from the chondrocytes (Howell 1975). This protease was inhibited by chloroquine. Teitz and Chrisman (1975) found that chloroquine inhibited the prostaglandin-induced destruction of rabbit articular cartilage. These authors felt that the action of chloroquine might be to inhibit the

synthesis of proteases. In light of the findings of other workers (Sapolsky, et al 1974), the action may be direct inhibition of the neutral protease. This distinction between the cathepsins and the neutral proteases may be regarded as merely a semantic one. The actions of the enzymes are similar, they merely have their activities at different pH ranges. Therefore the cathepsins can be regarded as acid proteases and conversely, the neutral protease can be regarded as a neutral or basic cathepsin.

With the identity of the protease established, we need to ask why it is present. There is no clear answer to that question. It has been proposed that the protease forms part of a system of continuous turnover of cartilage proteoglycan (Howell 1975). As suggested by George & Chrisman (1968), joint motion may give rise to matrix and cellular debris abraded from the surfaces and some mechanism is needed for removing it. The action of the protease could easily fit into such a scheme. Osteoarthritis may then represent a process where, for some reason, the protease becomes overactive, or that the proteoglycan-synthesizing capabilities of the chondrocytes become underactive, or some combination of both of the above.

We now need to consider what factors trigger off the proteolytic action mentioned above. Much work has been done in this area and the leading candidates

seem to be either the cyclic nucleotides or the prostaglandins. As will be discussed below, these 2 classes of compounds interact very closely and may turn out to be different components mediating the same sequence of events.

It is well-known that cholera endotoxin stimulates the production of cyclic adenosine monophosphate (cAMP). Prostaglandins also seem to have this property. Application of either cholera endotoxin or prostaglandin E_1 (PGE_1) to ileal mucosa causes inhibition of sodium absorption and stimulation of chloride secretion (Greenough, et al 1969, Al-Awqati, et al 1970).

Using the in vitro system to be described below, Fulkerson, et al (1978) showed that cholera endotoxin caused loss of proteoglycan from dog articular cartilage. In an analogous in vivo experiment, Floman, et al (1977) injected cell-free extracts of streptococci into rat knee joints and found a 3-fold rise in cathepsin D in the synovium. It has been suggested that endotoxins stimulate cAMP via a prostaglandin intermediate (Bennett 1971, Jacoby & Marshall 1972, Floman, et al 1977, Herman & Moncada 1975). In some of these experiments (Jacoby & Marshall 1972, Floman, et al 1977, Herman & Moncada 1975), a rise in prostaglandins could be measured, which could be prevented by addition of prostaglandin synthetase inhibitors such as indomethacin. This inhibition was accompanied by an inhibition of the

effects of endotoxins.

It should be noted, however, that this effect has not been universally demonstrated. Prostaglandin synthetase inhibitors failed to prevent endotoxin-induced lipolysis in isolated fat cells (Cuatrecasas 1973) or cAMP accumulation in human leukocytes (Bourne 1973). It is possible that endotoxins can directly stimulate cAMP production without going through prostaglandins.

A further complication is added by the work of Kuehl (1974; Kuehl, et al 1974), who showed, that, in some systems, there exists a post-cAMP pool of prostaglandins. This pool would be intra-cellular and would not be expected to respond to the action of prostaglandin synthetase inhibitors. Thus, he proposed a more complex modulating role of the prostaglandins rather than a straightforward stimulating role.

Despite the controversies surrounding the inter-relationship between the prostaglandins and cAMP, we may propose a tentative model for the pathogenesis of osteoarthritis (Figure 2). Any form of trauma results in some alteration of physical forces in the joint which, by some yet unknown mechanism, causes an increase in prostaglandins in the joint. In the case of the primary form of osteoarthritis, the pathogenetic sequence is assumed to begin here. The prostaglandins then stimulate cAMP, cause a release of proteases,

which then degrade cartilage proteoglycans and give rise to the typical histologic picture of osteoarthritis.

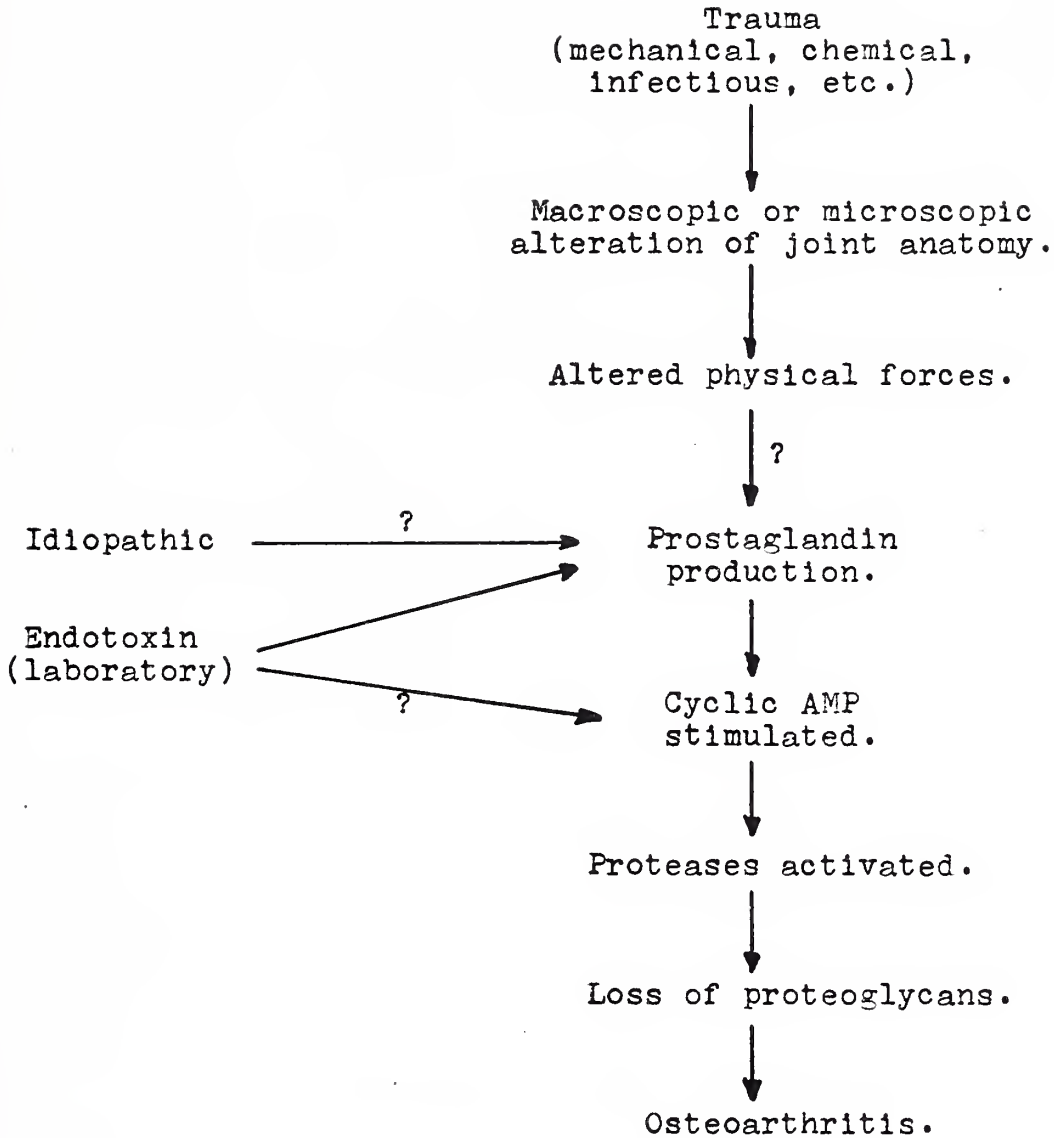


Fig. 2: A model for the pathogenesis of osteoarthritis.

Purpose

Previous work performed in this laboratory has demonstrated that prostaglandins can induce damage to rabbit knees, as shown by histological examination (Teitz & Chrisman 1975). Since then, an in vitro system has been developed in this laboratory whereby the actual loss of proteoglycan from the articular cartilage can be biochemically determined.

Briefly, the overall experimental method is as follows: Fresh articular cartilage is excised from dogs' knees and placed in a series of solutions containing the appropriate agents to be tested (prostaglandins, endotoxin, etc.). The cartilage slices are then recovered and the concentration of proteoglycans remaining are assayed by the method of Randle and Morgan (1955). By this method, the cartilage is hydrolyzed with concentrated hydrochloric acid. This breaks down the proteoglycan into hexosamines, the concentration of which can be determined spectrophotometrically.

Using this method, the prostaglandin-induced degradation of articular cartilage and the protective action of chloroquine has been demonstrated (Fulkerson, et al 1978). In addition, the system has also been employed to show the degradation of articular cartilage by cholera endotoxin (Fulkerson, et al 1978a).

The present study aims at demonstrating the

effects of arachidonic acid on articular cartilage. It has been shown that chondrocytes in cell culture are able to synthesize prostaglandins from arachidonic acid (Lippiello, et al 1977). If this is so, then introduction of arachidonic acid into the incubation medium of the cartilage slices should result in prostaglandin production and cartilage degradation. This would provide an in vitro model for part of the pathogenesis of osteoarthritis.

Furthermore, this would provide a useful system for the evaluation of the potency of prostaglandin synthetase inhibitors. Many such agents are used in the treatment of osteoarthritis. Indomethacin (Figure 3) is one of them and its clinical and pharmacological properties have been well described (Woodbury & Fingl 1975).

Sulindac is a new non-steroidal anti-inflammatory agent which has recently been released by the Food and Drug Administration for the treatment of arthritis. It is a congener of indomethacin and is claimed to have fewer side effects (BlanchiPorro, et al 1977, Lancet 1977). It has been speculated that the reason for the lower incidence of side-effects (mostly gastrointestinal) is related to its unique metabolism (Duggan, et al 1977). Sulindac, which is a sulfoxide, is metabolized by the liver to the sulfone and sulfide derivatives (Figure 4). The sulfone is readily excreted

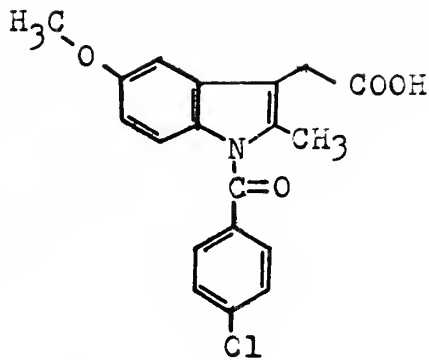


Figure 3: The chemical structure of indomethacin.

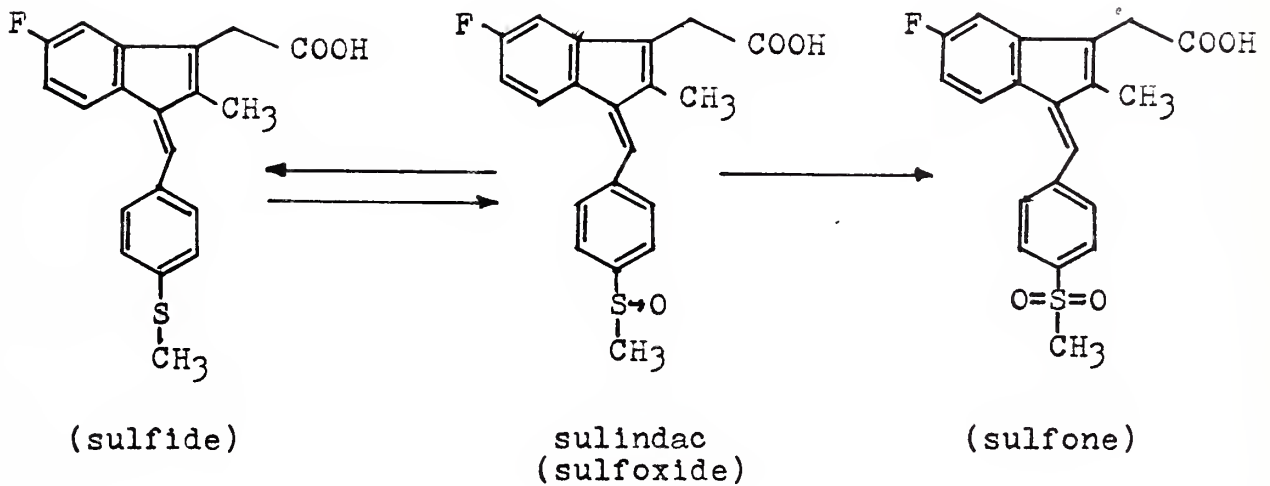


Figure 4: The chemical structures of sulindac and its sulfide and sulfone metabolites.

(From Duggan, et al 1977)

in the urine and has no pharmacological actions. The sulfide is not excreted in the urine (Shen 1976). It is many times more active than sulindac itself in its anti-inflammatory action in several in vivo models of inflammation (Duggan, et al 1977). Thus, it can be considered a "pro drug" with little action until metabolized by the liver and this may help explain the lower incidence of gastrointestinal side effects.

In the present work, the ability of the chondrocytes to synthesize prostaglandins from arachidonic acid will be shown by incubating cartilage slices in medium containing arachidonic acid and observing the loss of hexosamine from the cartilage. In addition, indomethacin and sulindac will be used to show that inhibition of prostaglandin synthesis will result in protection of the cartilage against hexosamine loss.

Methods

Preparation of solutions

1. Incubation solutions: Solutions were prepared immediately before use. Indomethacin* and the sulfide metabolite of sulindac** were dissolved in sterile lactated Ringer's solution in appropriate concentrations. Arachidonic acid*** was dissolved in absolute ethanol. Various quantities of each reagent and solvent were transferred into sterile 50-ml. Erlenmayer flasks stoppered with cotton wool plugs, such that the total volume of solution was always 6 ml.

2. Alkaline acetylacetone: Two ml. of acetylacetone (Fisher Scientific Co.) were dissolved in 0.5 N Na_2CO_3 to make 50 ml. of solution, and used not more than 2 hours after preparation.

3. Ehrlich's reagent: 0.8 gm. of p-dimethylaminobenzaldehyde (Fisher Scientific Co.) was dissolved in 30 ml. of absolute ethanol. To this was added 30 ml. of concentrated hydrochloric acid. The reagent was used within 2 hours.

* Binder-free indomethacin (L-590,226-00A116) was kindly supplied by F. A. Cutler, Ph.D. of Merck, Sharp and Dohne Laboratories, P. O. Box 2000, Rahway, New Jersey 07065.

** Binder-free sulfide metabolite of sulindac (L-612,835-00X25) was kindly supplied by Merck, Sharp and Dohne Laboratories, above address.

*** Na arachidonate from Sigma Chemicals, St. Louis, Missouri 63178. Lot no. 18C-7580.

4. Glucosamine standard solutions: Fourteen mg. of glucosamine HCl (Sigma Chemical Co.) were dissolved in 100 ml. of distilled water and kept refrigerated until used (less than a week).

Procedure

Fresh articular cartilage was obtained from adult dogs killed by intracardiac administration of pentobarbital immediately before surgery. The knees were shaved and prepped with Betadine scrub and alcohol and sterile technique was observed during the procedure. Each knee joint was exposed employing an anteriorly located vertical skin incision passing just medial to the patella. The joint was then entered with a horizontal incision through the anterior portion of the capsule, transecting both the medial and lateral collateral ligaments. Finally, the cruciate ligaments were transected and the menisci were removed to expose the articular surfaces. A fresh scalpel blade (#10) was then used to shave off pieces of articular cartilage. Most of the cartilage used in the experiments came from the femoral surfaces, with additional amounts from the tibial and patellar surfaces. Most slices measured 2 - 3 mm. X 5 mm. and were of full thickness. The slices were immediately introduced into the incubating solutions after pooling them in a Petri dish.

The flasks were incubated in an Eberbach

shaker bath at $37^{\circ}\text{C} \pm 1$ for 24 hours with moderate shaking (60 - 90 strokes per minute). At the end of this period, each flask was inspected for cloudiness suggestive of bacterial contamination. Suspicious solutions were sent for bacterial culture and, if positive, were discarded. Each sample was defatted and partly dehydrated by placing in 2 ml. of a 1:1 mixture of acetone and ethanol for 24 hours. The cartilage slices were recovered and the solvent completely driven off by placing in an evacuated oven at 60°C overnight. Two to 3 mg. of the dry cartilage was then weighed for the hexosamine assay.

The method for liberating the glucosamine was derived from the work of Boas (1953). Previous work done in this laboratory had shown that the best results were obtained (in terms of complete hydrolysis with minimal decomposition) by incubating the cartilage in 2 ml. of 4.25 N hydrochloric acid in 10-ml. volumetric flasks for 4 hours. At the end of this period, the solutions were topped to the 10-ml. mark with distilled water and filtered by gravity through Whatman no. 1 paper into a second set of 10ml. volumetric flasks.

At this point, blanks were prepared by pipetting into 10-ml. volumetric flasks 2 ml. of the same 4.25 N hydrochloric acid used in the hydrolysis and then topping to the mark. The standards were prepared by pipetting 0.5, 1.0, 1.5 and 2.0 ml. of the glucosamine HCl standard solution into 10-ml. volumetric flasks, add-

ing 2 ml. of 4.25 N hydrochloric acid and then topping to the mark with distilled water. The blanks and standards, however, were not filtered. From this point on, samples, blanks and standards were treated in an identical fashion.

The method employed for the quantitative determination of glucosamine was described by Rondle and Morgan (1955), which was a modification of the original method of Elson and Morgan (1933).

A 3-ml. aliquot from each flask was pipetted into a 10-ml. volumetric flask, titrated to the phenolphthalein end-point with 4 N NaOH and then back-titrated with 0.5 N HCl until the pink returned to colorless. Extreme care was necessary in this step since the assay is sensitive to both salt concentration and pH (Boas 1953). Next, 1 ml. of alkaline acetylacetone was added to each flask and heated in a boiling water bath for 30 minutes. The flasks were allowed to cool to room temperature. About 2 ml. of absolute ethanol were added, followed by 1.0 ml. of Ehrlich's reagent, in turn followed by sufficient ethanol to fill to the 10-ml. mark. The flasks were warmed to 65 - 70°C in a water bath for 15 minutes.

A Varian Series 634 spectrophotometer was used to read the absorbance at 530 nm. at a slit width of 1 nm. and a path length of 1 cm. All standards and samples were read at least twice against a blank refer-

ence and the average reading was used.

The standard curve was obtained using a linear least squares plot.

On most runs, the elapsed time between the addition of acetylacetone and the spectrophotometric determination was less than 6 hours. Similarly, the elapsed time between the addition of Ehrlich's reagent and final determination was less than 3 hours.

The data obtained was analyzed statistically using the student t-test of significance.

Results

The results of the experiments are represented in Tables 1 through 3. All values obtained are expressed in arbitrary units to facilitate comparison between different experiments. This is because, with the particular assay method employed, differences occur in the absolute values obtained from one experiment to the next, although they are internally consistent within each experiment (Rondle & Morgan 1955).

Table 1 shows the effect of arachidonic acid on the hexosamine content. When the concentration of the arachidonic acid in the incubating medium was 1 mcg/ml., no difference in the hexosamine content was noted between that and the controls. When the concentration of the arachidonic acid was increased to 10 mcg/ml., there was a significant ($P < 0.01$) drop in the hexosamine content from 100 to 72.6 arbitrary units. Further increasing the concentration of arachidonic acid to 100 mcg/ml. resulted in no change in the loss of hexosamine.

The effects of indomethacin on the system are shown in Table 2. The control values are set arbitrarily to 100. As expected, the presence of 10 mcg/ml. of arachidonic acid caused a drop in the hexosamine content, although the statistical significance of the results is not as good ($P < 0.10$ for Dog A, results insignificant for Dog B). Indomethacin alone did not cause

a change in the concentration, showing that it has no effect per se on the cartilage. The results of having both the indomethacin and arachidonic acid in the medium is slightly confusing. In the case of Dog A, the value obtained is half-way between that of the control value and that obtained with arachidonic acid alone. For Dog B, the value indicates a fully protective effect from the indomethacin. Since the sample size in the case of Dog A is small ($n=3$), one might expect it to be more easily influenced by random errors and would be less reliable. The sample size in the case of Dog B is bigger ($n=6$) and one would expect greater significance in the result. At any rate, Dog A still shows some protective effect rendered by the indomethacin.

In Table 3, the results of similar experiments, but using the sulfide metabolite of sulindac, are shown. Again, arachidonic acid causes loss of hexosamine. The values for Dog C are statistically insignificant while those for Dog D are ($P<0.05$). The sulfide metabolite of sulindac by itself causes no change in the hexosamine content. With both arachidonic acid and the sulfide metabolite of sulindac together, no change is noted in the hexosamine content, demonstrating the protective effects of the sulfide metabolite of sulindac, which is the active form of the drug.

Table 1: The effect of arachidonic acid on the hexosamine content of articular cartilage.

| Controls | Arachidonic Acid | | |
|----------------------------|------------------|---------------|---------------|
| | 100 mcg/ml | 10 mcg/ml | 1 mcg/ml |
| 100* (n=4) ⁺ | 75.6 (n=4) | 72.6 (n=4) | 98.9 (n=3) |

* Hexosamine concentration expressed in arbitrary units.

+ n = sample size.

Table 2: The effects of arachidonic acid and indomethacin
on the hexosamine content of articular cartilage.

| | Controls | Arachidonic acid 10 mcg/ml | Indomethacin 5 mcg/ml | Arachidonic acid 10 mcg/ml and Indomethacin 5 mcg/ml |
|-------|----------------------------|-------------------------------|--------------------------|---|
| Dog A | 100* (n=5) ⁺ | 80.7 (n=4) | 101 (n=4) | 89.8 (n=3) |
| Dog B | 100 (n=2) | 93.1 (n=3) | 102 (n=3) | 101 (n=6) |

* Hexosamine concentration expressed in arbitrary units.

⁺ n = sample size.

Table 3: The effects of arachidonic acid and the sulfide metabolite of sulindac on the hexosamine content of articular cartilage.

| | Controls | Arachidonic acid 100 mcg/ml | Sulindac (sulfide metabolite) 7 mcg/ml | Arachidonic acid 100 mcg/ml and Sulindac (sulfide metabolite) 7 mcg/ml |
|-------|----------------|--------------------------------|---|---|
| Dog C | 100* (n=3)+ | 89.5 (n=4) | 97.4 (n=3) | 99.5 (n=5) |
| Dog D | - | 84.8 (n=4) | 100 (n=4) | 99.8 (n=6) |

* Hexosamine concentration expressed in arbitrary units.

+ n = sample size.

Discussion

The foregoing experiments serve to demonstrate the effect of arachidonic acid on cartilage degradation. In all cases a drop in the amount of hexosamine is noted in samples containing the arachidonic acid (but without the prostaglandin synthetase inhibitors) as compared to controls which contained only lactated Ringer's solution and alcohol or which contained indomethacin or sulindac. Only low concentrations of arachidonic acid are necessary, as shown by the observation that increasing the concentration from 10 mcg/ml. to 100 mcg/ml. caused no further increase in hexosamine loss.

Furthermore, this not a direct effect of the arachidonic acid itself since if that were true, the indomethacin should not prevent the loss of hexosamine induced by the arachidonic acid. One might argue, on the other hand, that there may be a direct interaction between the indomethacin and arachidonic acid. The results are consistent with such a hypothesis as well. However, there is little evidence in the literature to support such a statement and there is much work to support the hypothesis that the action of indomethacin is by inhibition of prostaglandin synthesis (Vane 1971, Ferreira & Vane 1974, etc.).

Since chondrocytes are the only cells present in this system, they must be the source of prostaglandin synthetase. This agrees well with the previously-

mentioned work of Lippiello, et al (1977) who demonstrated that chondrocytes in cell culture were capable of prostaglandin synthesis. This system, moreover, has the chondrocytes in a milieu which much better approximates the in vivo conditions.

Another advantage of this system is that there is no interference from the enzyme systems from the much more cellular synovium. None of the work performed on in vivo systems can resolve the question as to whether the prostaglandins came from the synovial or cartilage cell synthesizing systems. This experiment shows that the latter are capable of producing the prostaglandins, although the question as to whether these prostaglandins or those originating from the synovium are ultimately more important in cartilage degradation. Certainly, in conditions such as rheumatoid or septic arthritis, intense synovitis is seen and the large amounts of prostaglandins detected in the joint fluid probably originate from there. However, the earliest osteoarthritic changes are detected in the articular cartilage and not in the synovium and it seems reasonable to conclude that, at least in the early stages, the prostaglandins are derived from the chondrocytes.

Although, in the experiments involving indomethacin and sulindac, the results are not as statistically significant, the trends shown by the results are quite clear. In all cases, the effect of the arach-

idonic acid in causing the loss of hexosamine from the articular cartilage cannot be demonstrated in the presence of either indomethacin or the sulfide metabolite of sulindac. The only exception is perhaps the case of Dog A, where the value for hexosamine loss with both indomethacin and arachidonic acid present fell roughly half-way between the values obtained for the controls and for arachidonic acid alone. However, the problem of the small sample size in that particular case has been discussed in the foregoing section.

The results are thus consistent with the model proposed above that the pathogenesis of osteoarthritis occurs via a pathway of endogenously-produced prostaglandins, leading to the synthesis or activation of proteases in the articular cartilage. It also shows that sulindac, a recently released non-steroidal anti-inflammatory agent and a chemical congener of indomethacin, also works by inhibition of prostaglandin synthesis.

One question which this experimental system is unable to address is the identity of specific prostaglandins synthesized. There are at least 6 products which may be formed from prostaglandin endoperoxide, eg. PGE's, PGF_{2α}, PGD, PGI, thromboxane. In rheumatoid synovial tissue in culture, the main product appears to be PGE₂, with some PGF_{2α} and PGB (Robinson & Levine

1974). Also, it is well known that different prostaglandins can have very different and even opposing actions (Vane 1971). Of note is the difference in actions between PGE_2 and $\text{PGF}_{2\alpha}$. In rat knees following the injection of cell-free extracts of Group A Streptococci, a rise in PGE_2 was noted within an hour while a rise in $\text{PGF}_{2\alpha}$ was noted only after 6 hours (Floman, et al 1977). Fulkerson, et al (1978a) used PGE_1 and PGE_2 in the present system and obtained cartilage degradation in both cases. Some have proposed that PGE_2 is an inflammatory prostaglandin while $\text{PGF}_{2\alpha}$ is an anti-inflammatory one (Willoughby & Dieppe 1976). Other workers, on the other hand, have postulated a pro-inflammatory action for $\text{PGF}_{2\alpha}$ (Weissmann, et al 1976). It would be useful as a next step to actually identify which of the prostaglandins are released during incubation with arachidonic acid and this would give important information on the more specific pathway of inflammation. From that point on, more specific prostaglandin synthetase inhibitors may be found which may have therapeutic advantages. For example, the gastrointestinal side-effects of indomethacin have been attributed to depletion of PGE_1 and PGE_2 in the gastrointestinal mucosa (Vane 1971, Robert 1974) which results in a breakdown of the mucus barrier. If it turns out that prostaglandins other than PGE_1 or PGE_2 are responsible for the osteoarthritic changes and specific inhibitors of these other prostaglandins can be

found, then the gastro-intestinal side effects of the present agents can be avoided.

Another point which should be considered in the light of these experiments is the actual effect of the non-steroidal anti-inflammatory agents in clinical use. Since the agents inhibited the degradation of proteoglycans, one could argue that, in clinical use, it should retard the progression of osteoarthritis, in addition to providing symptomatic relief. There is, however, no data available as to whether the non-steroidal anti-inflammatory agents actually alter the course of the disease. This is understandable since the clinical course of osteoarthritis varies tremendously and such long term follow-up studies would be virtually impossible to undertake.

As to the symptomatic relief of osteoarthritis, previous work has shown that PGE_1 or PGE_2 sensitized dogs to intra-articular injection of bradykinin (Moncada, et al 1975). The authors monitored blood pressure as a measure of pain and showed that aspirin or indomethacin prevented the effects of bradykinin but not those of the prostaglandins. Thus, they concluded that local prostaglandin synthesis sensitizes pain receptors to noxious stimuli. It has also been shown that incapacitation in dogs after intra-articular injection of endotoxin was highly correlated with the prostaglandin level in the synovial fluid (Herman

& Moncada 1975). Thus, prostaglandins mediate the symptoms of pain and incapacitation in the disease. It would seem reasonable to conclude that the prostaglandin synthetase inhibitors provide symptomatic relief by interfering in this pathway. There is no data at present to show whether they have any effect of articular cartilage preservation in osteoarthritis, but work on an analogous system has demonstrated such an effect. A study conducted on lateral dislocations of the patella may shed some light on this question (Chrisman, et al 1972, Chrisman & Snook, 1968). Patients were treated with a course of aspirin after being seen for the first episode of lateral dislocation of the patella. When dislocation recurred, surgical correction was recommended and the amount of chondromalacia judged by the system of Bentley (1970) when surgery was performed. In the untreated group, 21 out of 23 patients showed evidence of chondromalacia of any degree while this was true of only 3 of 16 knees in the aspirin-treated group. Since chondromalacia is an early finding in osteoarthritis, we have here an analogous situation which shows that aspirin in fact helps retard cartilage degradation in a clinical setting.

Possible sources of experimental error

Some of the values obtained in these experiments showed the trend quite clearly but the actual

numbers were not always statistically significant. It would seem appropriate to discuss some of the possible sources of error in the experimental system.

In the first place, there is some variation in the proteoglycan from animal to animal. (Maroudas 1975). The absolute amount of hexosamine cannot be meaningfully compared between animals and therefore arbitrary units have been employed. However, for each given animal, the results should be internally consistent. This is not possible due to a second variation, which is the variation in proteoglycan content as a function of depth (Lipshitz, et al 1976) and topographical location in the joint, as discussed before. Since the quantity of articular cartilage in each joint is extremely limited and all the cartilage which could be obtained had to be used, there was little which could be done to control for these two variables except by cutting the cartilage into very small slices, pooling the cartilage and randomizing the order by which the incubation flasks were filled.

The next step where a source of error is likely is the weighing of the dry cartilage. Most samples were between 2 and 3 mg. Since the balance reads to 0.1 mg., the accuracy of the weight is at best ± 0.1 mg., which when compared to the sample weight of 2 mg., is an error of at least 5 %. A larger sample size would lower this error but would give rise to the pro-

blem of incomplete hydrolysis.

It has been shown that hydrolysis of 0.5 mg. of purified chondroitin-6-sulphate with 1 ml. of 4 N hydrochloric acid under vacuum at 110°C was complete at 4 hours and shows a yield of greater than 90% at the end of 12 hours (Johnson, et al 1978). The problem we encountered was one of incomplete hydrolysis. After 4 hours, small flecks of black material were noted in the hydrolysate, which increased with the amount of cartilage used. The chemical nature of these flecks is unknown and therefore, the possibility of their containing an unknown amount of hexosamine cannot be ruled out. The error introduced in this step is indeterminate. Carrying on the hydrolysis for a longer period might circumvent this problem but we then run into the possibility of decomposition of the hexosamine.

Finally, Boas (1953) pointed out that many naturally-occurring substances contained chromogens other than hexosamines which would react to the reagents used to produce substances which absorbed at 530 nm. but with a different spectrum. Using a Dowex 50 quantitative ion exchange column, he was able to purify the hexosamines. However, such a procedure was not practical in this case because of the large numbers of samples used and the small volume in each sample. The error introduced here is again indeterminate. It can only be assumed that it was roughly the same in

each case and the relative values, which were the important ones in this experiment, were unaffected.

Summary and Conclusions

Previous work has demonstrated that prostaglandins can cause destruction of articular cartilage and that this may be part of the pathogenetic pathway of osteoarthritis. This paper seeks to elucidate this pathway by demonstrating the effect of arachidonic acid on articular cartilage. We shall then attempt to demonstrate that such an action can be inhibited by indomethacin and sulindac. The latter is a new non-steroidal anti-inflammatory agent and we shall show that its action is similar to that of indomethacin.

The results show that arachidonic acid caused a significant loss of hexosamines from articular cartilage. The trends of the results show that this effect can be inhibited by indomethacin or sulindac, which do so most likely by inhibition of prostaglandin synthesis.

Some of the possible sources of error in the experimental system which may have contributed to the lack of statistical significance in some of the numbers are discussed. Repeating the experiments using larger sample sizes may give more statistically significant results.

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